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Proliferative events experimentally induced by transient cold shock in the brain of adult terrestrial heterothermic vertebrates: preliminary analysis of PCNA expression in *Triturus carnifex*

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Summary

Experimental procedures used to investigate the persistence, location and abundance of scattered ("matrix cells") and/or clustered ("matrix areas") stem cells in the brain, responsible for proliferation in adult terrestrial heterothermic vertebrates have included an induced transient drop in body temperature in specimens subsequently deprived of encephalic areas. In a set of coordinated investigations focused on the influence of an exposure to a drastic thermally environment on these activities, we gave priority to *Triturus carnifex*, since there is a much larger amount of detailed, unequivocal experimental evidence available for this species than for other vertebrates of the same evolutionary level. In the present study, cold-shocked newts were examined after a stay at external temperature (the most suitable one based on previous experience) to allow the maximal expression of cerebral proliferation. In a qualitative evaluation, the brain of experimental specimens compared with that of normal individuals seemed not to show, contrary to expectations, more pronounced cell proliferation as assessed by Proliferating Cell Nuclear Antigen immunolabelling of neural-like cells in the S phase of cell cycle. This discrepancy with previous reports from other authors may depend on having used cold stress alone, while other traumatic stimuli (operatory shock, encephalic injury) administered by the previous authors might have induced a greater number of cells to move from a stand-by condition to proliferation, allowing for reparative and/or regenerative phenomena.

Key words

Adult newt; brain; cold shock; PCNA expression.

Introduction

It is commonly accepted that the vertebrate brain is endowed with reparative or regenerative power in the early stages of development (Cowan and Finger, 1982) and that the processes of self-repair are considerably reduced when the brain is fully developed. Nevertheless, a great amount of literature refer the persistence of cellular proliferative potential in the brain of adult Anamnia (lampreys, Elasmobranchs, Teleosts, Urodeles, Anurans) and Amniota (lacertilians, male songbirds, Mammals) (for

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details, see review by Margotta and Morelli, 1996), under both normal and experimental conditions and with different locations and degrees.

These studies were mostly autoradiographic and histological (sometimes ultrastructural). They only rarely used immunocytochemical tests or histochemical methods targeting proliferation-related enzyme activities. The study of these vertebrates has revealed a neurogenic ability depending on small basophilic cells which have retained some embryonic properties. They are clustered and at times stratified ("matrix areas" or "matrix zones") in several encephalic areas (mostly in the ependymal epithelium and/or periventricular grey matter) or scattered ("matrix cells") in the tissue of other cerebral regions. Generally speaking, these neural-like cells are more numerous in lower than higher vertebrates and in younger than older animals. They appear able to return to an actively cycling condition and to undergo proliferation through late maturation even in the absence of these events, to give rise to neuronal and glial cells (Kirsche, 1967, 1983).

In the adult vertebrate brain, possible relationships between the presence of radial glial cells and the persistence of natural or post-traumatic cell proliferation have emerged from critical reviews on these topics (Margotta and Morelli, 1997; Alvarez-Buylla et al., 2002). On the basis of selective autoradiographic or immunocytochemical tests, a double function has been hypothesized for such cells: first favor the production of neuroblasts from cells in a stand-by condition and then guide the migration of the newly formed neurons to their definitive sites. It is interesting that the radial glia cell bodies are in the same position as the *zonae germinativae*, as first indicated by Kirsche (1967).

Over the last decade, we have undertaken an immunocytochemical re-evaluation of the cellular proliferative capacities of the brain in normal adult specimens belonging to each Class of heterothermic vertebrates and songbirds (Margotta et al., 1999a, b, 2000; 2001, 2002, 2004, 2005a, b; Margotta and Caronti, 2005; Margotta et al., 2007; Margotta, 2007) and in the same or phylogenetically similar species as those previously analyzed by other authors, for a valid comparison. We have observed a substantial agreement between our findings and literature reports, minor differences being compatible with the higher age of our individuals.

At the end of such investigations we concluded that: "in an overall view … the matrix cells are always identifiable in the epithelium of ventricular walls, in the periependymal grey layers and in the framework of cerebellar tissues, when the *cerebellum* carries out a leading role in some essential activities for the life and it's involved in the social behaviour of the organism" (Margotta, 2007). This is similar to what Kirsche (1983) had stated in his review.

The various experimental procedures used in the past in terrestrial poikilothermal vertebrates to detect regenerative events in injured brain include exposure of the organism to a sudden, transient drop in body temperature.

Del Grande and Minelli (1971) and Minelli and Del Grande (1974a, b) were the first to introduce this cold shock. They observed an unexpected increase of regenerative events in adult *Triturus cristatus carnifex* that had previously lived temporarily in cold conditions and was then surgically deprived of a portion of the brain.

Later, the hypothesis was proposed that a thermal shock might be linked to a cellular proliferative response and that it might in some way stimulate latent cells to undergo late maturation processes.

Further investigations were performed by a research group on adult *Lacerta viridis* (Minelli et al., 1978; Minelli and Del Grande, 1980; Del Grande et al., 1981), *Rana esculenta* (Minelli et al., 1982; Del Grande et al., 1984) and *T. cristatus carnifex* (Del Grande et al., 1982a, b; Minelli et al., 1987; Del Grande et al., 1990; Minelli et al., 1990; Franceschini et al., 1992). Most of these studies were carried out by histology and autoradiography and a few by electron microscopy, after thymidine injection or treatment with colchicine respectively. The undifferentiated ependymal and sub-ependymal cells observed in the telencephalon, diencephalon and mesencephalon were deemed responsible for substantial and unexpected self-repair processes leading to the complete structural restoration of the removed portion of the brain (Del Grande and Minelli, 1971; Minelli and Del Grande, 1974a, b; Del Grande et al., 1982a, b; Minelli et al., 1987; Del Grande et al., 1990; Minelli et al., 1990; Franceschini et al., 1992; see also the reviews by Kirsche, 1983; Margotta and Morelli, 1996).

Since in the last decades the attention on this issue has waned, this has encouraged us to undertake a new study in adult terrestrial heterothermic vertebrates, focusing once again on encephalic cell proliferation, now induced by a transient thermal stimulus. Like in our above-mentioned studies, these animals would not be subjected to any cerebral mutilation in order to evaluate whether the mere artificial temperature change can promote an increase in proliferation. One reason for this study is that observations concerning this specific aspect were sporadic and restricted to a small number of individuals of *R. esculenta* (Minelli et al., 1982) and *T. cristatus carnifex* (Franceschini et al., 1992).

We have focused on *T. carnifex* (Giacoma and Balletto, 1988; Bonifazi, 2000), *R. bergeri* (Capula, 2000) and *Podarcis sicula* (Capula, 2000) (formerly, *T. cristatus carnifex*, *R. esculenta*, *L. viridis*, respectively), i.e. the same species studied by previous authors, and have used the same test, PCNA immunolabelling (Miyachi et al., 1978), which had proved valuable in our former investigations.

Proliferating Cell Nuclear Antigen, an ubiquitous intracellular antigen and member of the cyclin family, is an auxiliary protein of DNA polymerase δ closely associated with sites of DNA replication. It reaches appreciable levels when DNA is synthesized in the cell cycle, thus revealing cells in S phase (Bravo and Macdonald-Bravo, 1985, 1987; Bravo et al., 1987; Jaskulski et al., 1988; Liu et al., 1989; Fairman, 1990; Diffley, 1992; for further details: see Margotta et al., 2007).

The experimental conditions adopted in previous investigations focused on cold shock by other authors were certainly unequivocal about the degree of temperature, while the other reported information was often conflicting, defective or ambiguous on how the temperature change was attained: whether rapidly or gradually (Del Grande and Minelli, 1971), how protracted in time (Del Grande and Minelli, 1971; Minelli and Del Grande, 1974a, b), at which temperature the experimental samples were housed after the thermal treatment (Del Grande et al., 1981; Franceschini et al., 1992) and how many days after the shock the individuals were sacrificed (Franceschini et al., 1992); in some instances there was complete lack of information on some of these aspects (Minelli and Del Grande, 1980; Del Grande et al., 1982a; Minelli et al., 1982).

T. carnifex was given priority in our investigation since a much larger amount of detailed information is available for this species (Del Grande et al., 1982a; Minelli et al., 1987; Del Grande et al., 1990; Minelli et al., 1990; Franceschini et al., 1992) than for the frog or lizard. We have used the experimental conditions most frequently

reported in the literature, but without removal of part of the brain. However, previous reports did not state when the newts were taken from their habitat or when the observations were carried out.

Material and methods

Adult specimens of *Triturus carnifex* (Giacoma and Balletto, 1988; Bonifazi, 2000) of both sexes were caught in the second week of March in a temporary pond in a coastal area of Latium, Italy. This period corresponds to the breeding season of this species (Bonifazi, 2000). The external temperature in this period can vary from 7 to 17 °C.

The newts were separated into two groups: those in the first group continued to live in this environment, while those in the second group were kept at 4 °C (temperature reached abruptly) for 24 hours, after which they were returned outside. After one week, the newts of both groups were sacrificed after anesthesia with a solution (1:1000) of tricaine-methanesulfonate (MS 222 Sandoz, Switzerland). The heads were then cut off and, after partial disarticulation of the cranial elements, fixed in Bouin's fluid. During immersion of the heads in 80% ethyl alcohol, the brain was removed under a stereomicroscope. After dehydration in an ascending alcohol series the brains were embedded in paraffin wax in a vacuum. The histological preparations were made using 8 μm thick transverse serial sections cut in an antero-posterior direction with a rotary microtome. Two parallel series of slides were obtained for each brain: one was stained with hematoxylin-eosin, while the other was used for immunocytochemical examination.

Sections were heated in an oven at 60 °C for 20 min until the paraffin melted. The slides were deparaffinized and rinsed in 100%, 95% and 70% ethanol. A Vectastain Universal Quick kit (Vector Labs, Burlingame, CA, USA) and 0.01 M phosphate buffer, pH 7.5, with 0.02% Triton X 100 as washing buffer were used. The procedure steps, at room temperature, were: 10 min in 3% H_2O_2 solution in water, 5 min rinse, 10 min in blocking serum, 15 min + 15 min in avidin/biotin blocking kit (Vector Labs), brief rinse, 90 min in the monoclonal antibody to PCNA clone (Sigma, Milan, Italy; cod. P8825), diluted 1:500 in buffer containing 1.5% blocking serum, 5 min rinse, 10 min in biotinylated universal secondary antibody, 5 min rinse, 10 min in streptavidin/peroxidase complex, 5 min rinse, 10–15 min incubation in Nova Red or DAB substrate kits (Vector Labs), with or without nickel enhancement. The sections were then washed and mounted in Kaiser's glycerol gelatin (Sigma). Control sections of representative tissues were prepared by substitution of the primary antibody with dilutions of normal mouse serum or omission of the primary antibody. A section of regenerating rat liver, in which a high cell proliferative activity had been documented by incorporation of bromodeoxyuridine, was used as positive control tissue.

Results

In *T. carnifex* adults, seven days after exposure to transient cold shock the olfactory bulbs display a large number of immunolabeled cells at the boundary of the ventricles, in the sub-ependymal grey matter and in the internal granular layer (Fig. 1a).

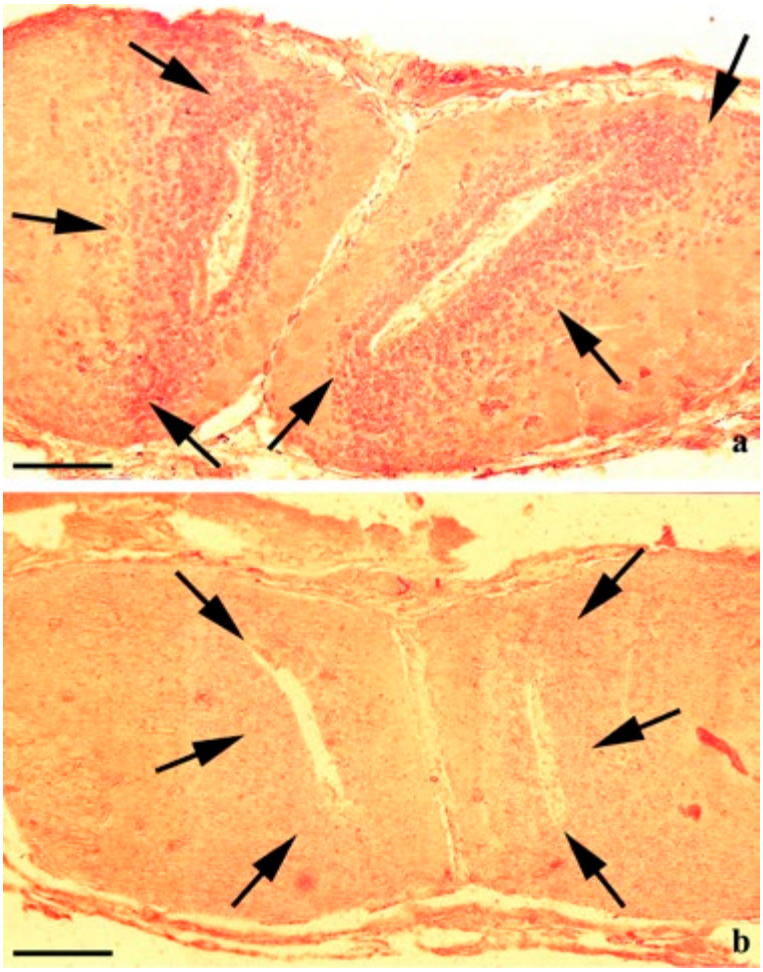

Figure 1 – In the olfactory bulbs of an adult *Triturus carnifex* stem cells are visible in the ependyma, subependymal grey matter and internal granular layer (arrows): a) specimen exposed to cold shock; b) normal specimen. Transverse sections, PCNA immunocytochemistry without nuclear counterstaining. Calibration bars = 60μm.

In adults of the same species not subjected to cold stimulus, these areas show fewer immunopositive cells (Fig. 1b).

In the telencephalon of cold-shocked individuals, small clusters of PCNA-labeled cells are placed anteriorly in the proximity of the outer corners of the roof of each hemispheric ventricle (sometimes swollen) exactly where the *area germinativa dorsalis* (formerly called *zona germinativa dorsalis* by Kirsche, 1967) lies. The number of these cells decreases moving caudally until they disappear approaching the telencephalic intermediate portion. Posteriorly, while such immunoreactive aspects reappear, substantial DNA synthesis can be observed in the external lower walls and on

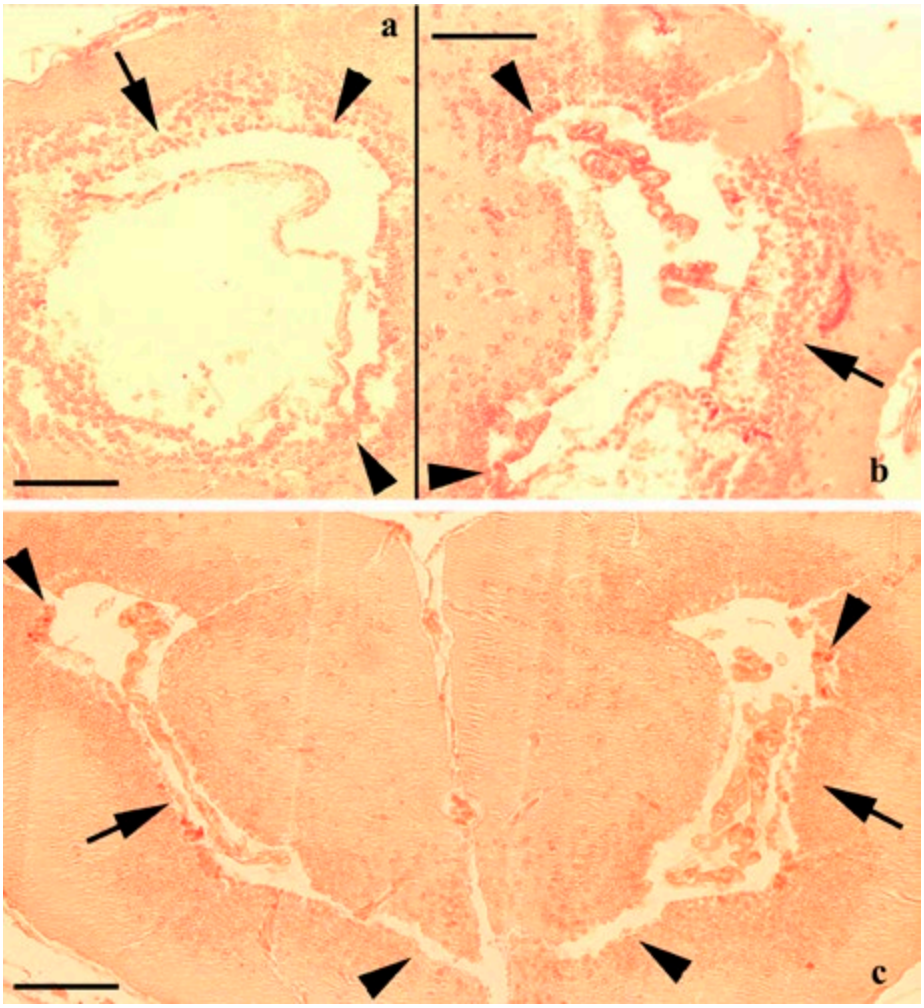

Figure 2 – In the telencephalon of an adult *Triturus carnifex* PCNA-positive cells are found in the *areae germitivae dorsales* and *ventrales* (arrowheads), in the ventricular epithelium and in the periependymal layer (arrows): a) left hemisphere, and b) right hemisphere in a specimen exposed to cold shock; c) both hemispheres in a normal specimen. Transverse sections, PCNA immunocytochemistry without nuclear counterstaining. Calibration bars = 60µm.

the floor of the ventricles. The site of this PCNA-positivity coincides with the location of the *area germinativa ventralis* (*zona germinativa ventralis* according to Kirsche, 1967). Proceeding caudally, while the dorsal immunocytochemical labeling decreases and definitively disappears, parallel to the progressive disappearance of the *area germinativa dorsalis*, the labeling continues where the lateral ventricles merge and the *area germinativa ventralis* is found. Other proliferative patterns appear here and there in the ependyma of these regions. Widespread, weak PCNA expression can also be

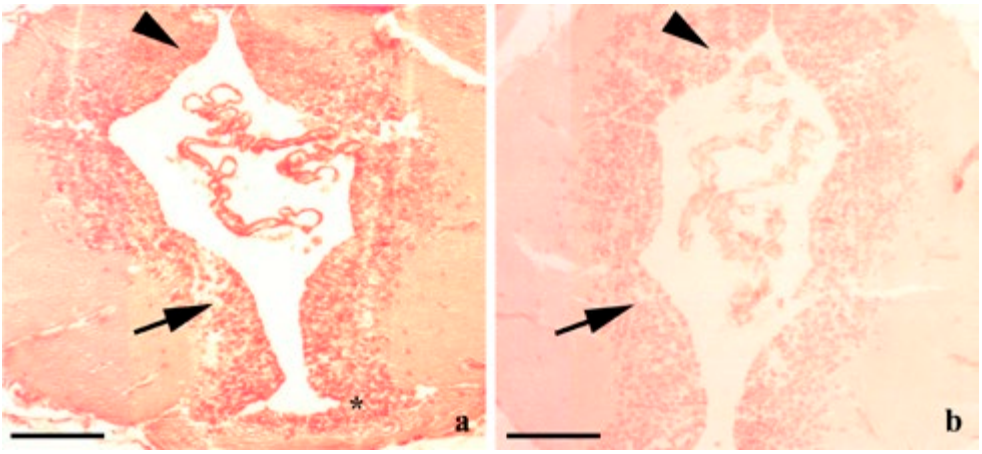

Figure 3 – In the diencephalon of an adult *Triturus carnifex* undifferentiated cells are present in the habenular ganglia (arrowheads), pre-optic recess (asterisks), ventricular epithelium and sub-ependymal layer (arrows): a) specimen exposed to cold shock; b) normal specimen. Transverse sections, PCNA immunocytochemistry without nuclear counterstaining. Calibration bars = 60µm**.**

observed among the cells populating the layers adjacent to the epithelium delimiting the ventricular cavities and the telencephalon impar (Fig. 2a, 2b). Also in the normal telencephalon, immunolabeled cells are identifiable in the *areae germinativae dorsales* and the *areae germinativae ventrales*. These cells can be observed in the ependymal epithelium and periventricular grey matter (Fig. 2c).

In the diencephalon of cold-shocked newts, labeled cells are present in the habenular ganglia and frequently among the epithelial cells which delimit ventricle in the epithalamus, thalamus and preoptic and infundibular recesses in the hypothalamic region; in the latter site they are especially numerous. Immunocytochemical expression can also be seen in the grey matter of these regions (Fig. 3a). In control individuals, PCNA positive cells are seen in the same areas (Fig. 3b).

In the mesencephalon of the cold-shocked specimens, labeled cells are visible in the ependymal epithelium facing the optic lobes and the *tegmentum.* A substantial immunocytochemical reaction also occurs in the peri-ependymal grey matter. Rare scattered or thickened PCNA-positive cells are found here and there among the cells in the deep and intermediate layers of the optic *tectum* (Fig. 4a). In the normal midbrain, similar aspects can be observed in the ventricular epithelium, sub-ependymal grey matter and *tectum* (Fig. 4b).

The *cerebellum* does not appear labeled in either experimental or control specimens.

In the *medulla oblongata* of cold-shocked newts, there are an immunoreactivity in the *area octavolateralis* and a small number of labeled cells in the floor of the anterior portion of the rhombencephalic ventricle (Fig. 5a). In control specimens, similar PCNA expression appears in the same areas (Fig. 5b).

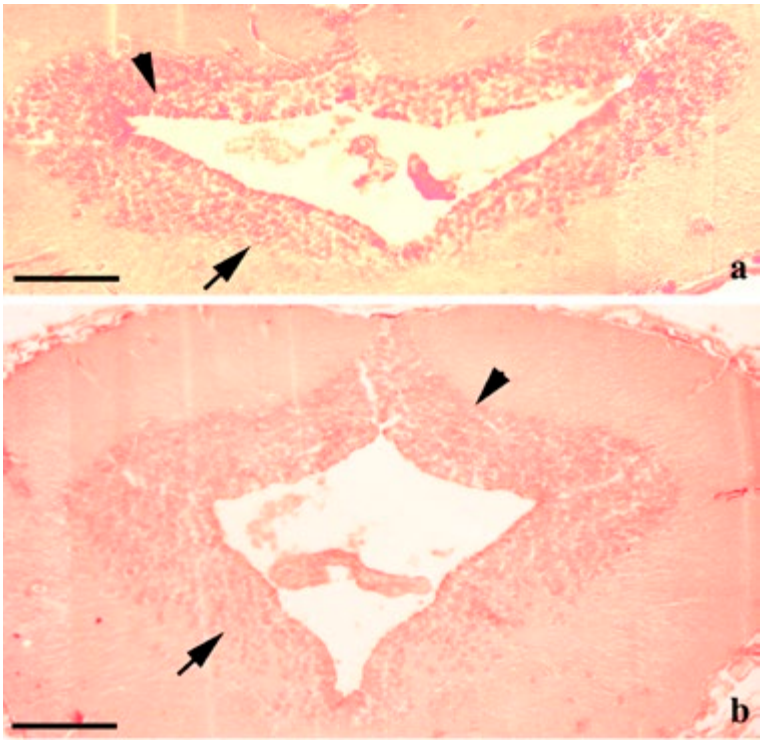

Figure 4 – In the mesencephalon of an adult *Triturus carnifex* labeled cells are visible in the ependyma and grey matter of the tectum (arrowheads) and *tegmentum* (arrows): a) specimen exposed to cold shock; b) normal specimen. Transverse sections, PCNA immunocytochemistry without nuclear counterstaining. Calibration bars = 60µm**.**

Discussion

Evidence for the encephalon of adult terrestrial poikilothermal vertebrates indicates that the number of cells in stand-by is high in the forebrain, while the hindbrain is deficient or laking of these cells.

Del Grande and Minelli (1971), Minelli and Del Grande (1974a, b) found an unexpected increase in encephalic plasticity in adult individuals of *T. cristatus carnifex* after transient cooling at 4 °C (aimed at limiting cardiac activity thus reducing postoperative hemorrhage and consequent mortality) followed by unilateral removal of a plug from the *optic tectum.*

Investigations on thermal-shocked individuals of this same species upon unilateral surgical ablation of cerebral areas have demonstrated that, if the damage regarded both the diencephalon and midbrain (Del Grande et al., 1982a) or both the telencephalon and midbrain (Del Grande et al., 1990; Franceschini et al., 1992), tangible regenerative phenomena occurred also in the diencephalon and mesencephalon which, when subjected to lesion alone, were not characterized by regeneration. It has been hypoth-

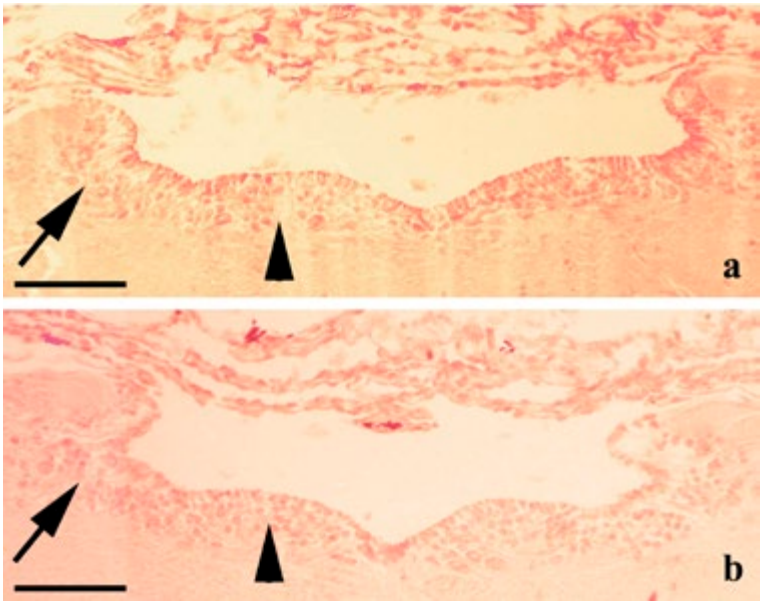

Figure 5 – In the *medulla oblongata* of an adult *Triturus carnifex*, neuroblasts labeled cells can be observed in the *area octavolateralis* (arrows), in the epithelium facing the ventricle and in the grey matter (arrowheads): a) specimen exposed to cold shock; b) normal specimen. Transverse sections, PCNA immunocytochemistry without nuclear counterstaining. Calibration bars = 60µm.

esized that a neurotrophic factor, secreted from a damaged tissue and released in the cerebrospinal fluid, could induce proliferation in the silent cells, placed far-off from the site of the lesion. In fact, the proliferation observed in the lateral ventricular walls, owing to an optic lobe injury, could be due to the matrix cells located in the periventricular layer and therefore easily attainable by chemical agents present in the fluid.

On the contrary, a factor produced by the edges of a telencephalic wound could not be able to induce proliferation in the midbrain where the matrix cells are scattered in the deep tectal grey layers.

A protein factor was found in the cerebral damaged tissues of adult rats, which stimulated *in vitro* a neuronal proliferation (Nieto-Sampedro et al., 1982).

Confirmation of the results also came from investigations upon ablation of the same areas in cold-shocked adults of *L. viridis* by Minelli et al. (1978), Del Grande and Minelli (1980), Minelli and Del Grande (1980), Del Grande et al. (1981), of *R. esculenta* by Minelli et al. (1982), Del Grande et al. (1984) and of *T. cristatus carnifex* by Del Grande et al. (1982b), Minelli et al. (1987, 1990), Franceschini et al. (1992). In some collateral investigations a handful of specimens were exposed to cold shock alone without removal of any brain portion (Minelli et al., 1982; Franceschini et al., 1992).

The results of the latter studies supported the initial observations of Del Grande and Minelli (1971), Minelli and Del Grande (1974a, b), leading to the hypothesis that low temperature loosens the blood-brain barrier, which could stimulate or support in adulthood the re-establishment of embryonic hemotrophic conditions (Del Grande et

al., 1982a). Cold stress could stimulate karyokinesis activity in matrix cells still present in encephalic areas, triggering the ability of these stem cells to originate new nerve cells and favoring the reappearance or substantial boosting of cerebral tissue plasticity, as evidenced by experimentally induced reparative and/or regenerative phenomena.

This hypothesis is supported by literature data on Anamnia and Amniota (Rosomoff and Gilbert, 1955; Stone et al., 1956; Lougheed et al., 1960; Kienan, 1979; Kienan and Contestabile, 1980), according to which low temperature leads to opening the blood-brain barrier, which is practically non-existent in early embryonic stages, is imperfect until morphogenesis has been completed, and is fully formed only at the end of development. Thus, it may be proposed that nervous tissue proliferation, expressed to a remarkable extent during embryonic life, declines during development owing to formation of the blood-brain barrier. Re-established permeability would accompany, or even determine, the restoration of reparative and/or regenerative processes in the central nervous system.

Cyclic seasonal thermal variations might induce fluctuations in cell proliferation in adults, as suggested by data on *R. esculenta* (Rothstein et al., 1975; Minelli et al., 1982; Bernocchi et al., 1990; Chieffi Baccari et al., 1994), *R. temporaria* (Chetverukhin and Polenov, 1993; Polenov and Chetverukhin, 1993), *P. hispanica* (Ramirez et al., 1997) and *Plethodon cinereus* (Dawley et al., 2000).

Links between photoperiod, temperature and physiological cell proliferation have been observed in other Anamnia such as the teleost *Tinca tinca* (Velasco et al., 2001) and the lamprey *Petromyzon marinus* (Vidal Pizarro et al., 2004). Some of these authors observed peak mitotic activity not only in the brain, but also in other organs and tissues, such as the eye (Rothstein et al., 1975), chemosensory epithelium (Dawley et al., 2000) and spinal cord (Velasco et al., 2001), of specimens captured between late spring and early summer, while a drop in proliferation was observed in individuals caught during hibernation.

These reports have led to the conviction that seasonal thermal variations, which are correlated with photoperiod, and artificially induced cold shocks can cause fluctuations in proliferative activity in adult heterothermic vertebrates.

Our present results cannot be directly compared with previous investigations because of differences in the experimental approach. Indeed, in *T. cristatus carnifex* adults, Del Grande and Minelli (1971), Minelli and Del Grande (1974a, b), Del Grande et al. (1982a, b), Minelli et al. (1987), Del Grande et al. (1990), Minelli et al. (1990) and Franceschini et al. (1992) judged cell proliferative performance from reparative or regenerative events upon transient cold shock followed by cerebral mutilation. On the contrary, Franceschini et al. (1992) performed autoradiographic analyses upon cold stress alone and on a limited number of specimens.

Nor can the present results be compared with those on *T. carnifex* from our previous immunocytochemical studies since they were performed on normal individuals also captured in early spring, a period corresponding to the breeding season (Bonifazi, 2000), but then maintained for a long time in a thermostatically controlled environment before sacrifice (Margotta et al., 1999, 2005b).

The presence of a telencephalic ventricular swelling in our thermal-shocked specimens, as compared with normal newts, may depend on cold environment which may provoke a decrease in cell number through cell death (which is often seen in association with ependymal and sub-ependymal neurogenesis, even if the brain is not sub-

jected to surgical injury). It has been shown that thermal stress and other insults (for instance, alterations in cerebral blood flow, blood pressure or blood gases) can affect even Mammals.

On the basis of these literature data we had supposed that upon cold shock we would find higher PCNA labeling than normal; on the contrary we did not find substantial differences.

Since our newts, after the temporary stay in a drastic thermally environment, were transferred to the external temperature in the range most suitable to express the maximal level of brain cell proliferation (Franceschini et al., 1992), the present findings might be imputable to the impact of lowered body temperature alone on the latent proliferative capacities still present in adulthood.

The additional traumatic stimulus (surgical injury and cerebral ablation), put into action by previous authors, might have induced more cells in stand-by to undergo proliferation, leading to the reparative and/or regenerative processes described by those authors.

Future research will address in detail the features of stem cells and their putative destiny towards glial cells or neurons, as well as the extent of apoptosis and of key proteins possibly involved in thermal exposure response.

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